HYPEROXIA Responses and ROS

9/10/24

NCT05958303

Title: Human cerebral blood flow regulation: sex, mechanism, and stress differences

Phase 3: HYPEROXIA responses and role of oxidative stress

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Funding Sponsor(s): National Institutes of Health (NIH)

Contents

I. Abbreviations/Acronyms

1. CBF: Cerebral Blood Flow
2. CVC: Cerebrovascular Conductance, CVC=CBF/BP
3. BP: Blood Pressure
4. MABP: Mean Arterial Blood Pressure
5. HR: Heart Rate
6. SpO_2: Oxygen saturation by pulse oximetry
7. $ETCO_2$: End-Tidal CO_2 (measured in exhaled breath)
8. Hyperoxia: Condition where participants breathe 100% oxygen (medical grade) resulting in 99-100% SpO_2 saturation levels in blood. Easily reversible when returned to room air breathing.
9. ROS: Reactive Oxygen Species. A broad term for many free radicals. ROS is associated with many cardiovascular diseases, and ROS levels acutely increase during exposure to hyperoxia (excess oxygen).
10. AOC: Antioxidant Cocktail. Formulation of oral over the counter supplements. While taken by many adults as part of a healthy diet, these are also shown to scavenge ROS.

II. Key Personnel

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1. PROJECT SUMMARY

The risk of cerebrovascular disease is inversely associated with resting cerebral blood flow (CBF). Men exhibit a lower resting CBF and have twice the risk of cerebrovascular disease when compared to premenopausal women (Appelros, Stegmayr, & Terent, 2009; Mensah, Mokdad, Ford, Greenlund, & Croft, 2005). The ability of cerebral vessels to respond to challenges is also inversely related to disease risk and may be useful in identifying at-risk patients pre-clinically (Lavi, Gaitini, Milloul, & Jacob, 2006; Matteis, Troisi, Monaldo, Caltagirone, & Silvestrini, 1998; Rogers, Meyer, Mortel, Mahurin, & Thornby, 1985; Silvestrini et al., 2006). However, these studies are often confounded by aging and/or comorbidities, and the associations provide little insight into physiologic mechanisms responsible for sexually dimorphic cerebrovascular disease risk. Conversely, animal studies use supraphysiologic levels of hormone treatment in primarily young animals, which limits the translational relevance of animal CBF mechanisms (Gonzales, Duckles, & Krause, 2009; Krause, Duckles, & Gonzales, 2011; McNeill, Kim, Duckles, & Krause, 1999). While there is general agreement that estrogen is protective in healthy adults, the basic impact of sex, and physiologic fluctuations in sex hormones, on mechanisms of CBF control remains unclear (Gonzales et al., 2009; McNeill et al., 1999). The overall goal of this research program is to investigate the mechanisms which actively control CBF in humans, particularly how men and women differ in control mechanisms on a regional basis throughout the brain circulation during physiologic stressors. We propose to study CBF control mechanisms in healthy younger (18-40 yrs) adult men and women. This subproject focuses on high oxygen exposure (hyperoxia). These exposures are common in clinical settings with subjects receiving high flow oxygen as well as military applications such as jet pilots. While hyperoxia in these states is used for organ health (clinically) and to avert crisis for pilots in case of rapid decompression (and loss of consciousness), there are likely negative aspects to hyperoxia. In particular, hyperoxia promotes production of Reactive Oxygen Species (ROS), typically as an unpaired electron on oxygen (superoxide) that leads to cellular damage. Literature suggests sexes differ in their antioxidant responses to ROS. Broadly speaking, females and higher estrogen levels are associated with lower ROS production and/or higher ROS scavenging (antioxidant response). To our knowledge, no human studies have directly tested the CBF response to hyperoxia between males and females.
The overall hypothesis is that males will demonstrate greater reductions in CBF in response to hyperoxia compared to females, due to greater influence of ROS. To test this hypothesis, healthy younger adults (18-40 yrs) with no comorbidities will complete two MRI study visits where they are exposed to hyperoxia in a randomized placebo-controlled design. We will acutely manipulate ROS by having the subjects ingest an antioxidant cocktail (AOC) shown to scavenge or reduce ROS. Based on our main hypothesis, hyperoxia will reduce blood flow in both sexes, but the AOC will reduce the hyperoxic vasoconstriction (thereby increasing the CBF) more in males, since females have more endogenous antioxidant signaling. CBF will be quantified by high-resolution, flow sensitive MRI to quantify CBF at rest, and in response to hyperoxia. These novel, high resolution, regionally-specific, sex-specific findings will serve as a knowledge platform for designing sex-specific therapies in high-risk disease populations which exhibit strong sex-specific etiology with important vascular contributions.

2. SIGNIFICANCE

2.1. Synopsis

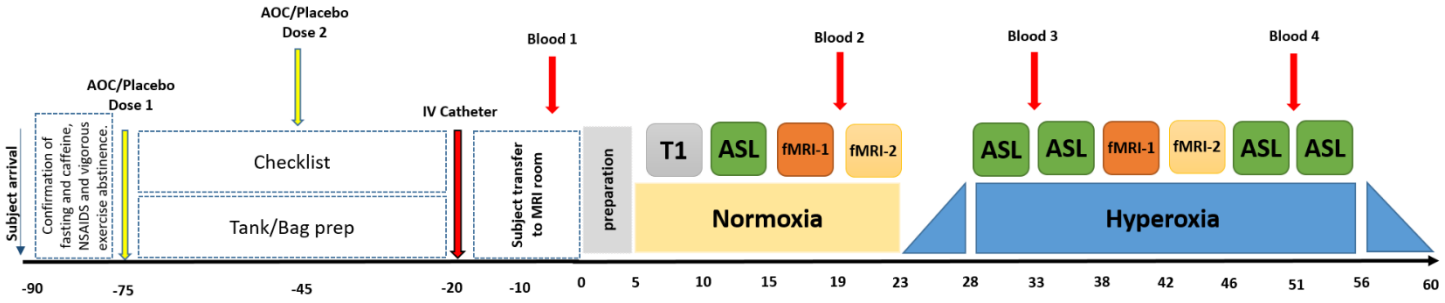
Objective: The **current objective** is to determine whether biological sex influences CBF control in hyperoxia in healthy young adults without confounds of age or disease. We will address 3 specific questions: 1) Are cerebral vasoconstrictor responses to hyperoxia greater in men? 2) Do all brain regions respond equally, or are there regional differences—possibly varying by sex? 3) Do ROS regulate the *decrease* in CBF in a sex specific fashion? This study will be conducted in compliance with federal investigational drug regulations (21 CFR 312) and Good Clinical Practice (GCP) guidelines, as well as state law and institutional policies.

Study Population: This study includes 30 total participants; (15 males and 15 females) who are ≥18 - ≤ 40 years old and considered healthy.

Approach: CBF testing will be performed in research-dedicated MRI systems on UW campus. Subjects will experience normoxia followed by hyperoxia conditions during each of the 2 study visits. Study design focuses on the use of an acute oral antioxidant cocktail (AOC) to test ROS signaling as a potential mechanism explaining sex differences in CBF control. To do this, we will conduct 2 MRI visits in a double-blind placebo-controlled design. (See Overview Figure below)

Phase 3: MRI visits:
See figures below.

Figure 1: Overview of Study



Prior to the first study visit, the order of placebo/antioxidant supplementation will be randomized. The subject will receive either just the placebo or just the antioxidant during each study visit. The selection of the placebo and antioxidant will be randomized and double-blind. Two MRI visits will be scheduled based on MRI and subject availability. Procedures are identical in both MRI visits with the exception of placebo/antioxidant.

Study Drugs:

- Oral antioxidant cocktail (AOC) to scavenge ROS signaling
  - By design, subjects will receive AOC during a randomized study visit.
  - The study team will give the subject the first AOC dose (3 pills) 90-120 minutes prior to entering the MRI suite and then dose #2 (3 pills) as a booster ~60 mins prior to entering the MRI suite. Based on pharmacokinetics and on numerous prior studies in humans, this period robustly decreases blood markers of ROS.
- Placebo
  - Placebo pills similar in size and shape will be administered to the subject. These typically contain inert safe ingredients.

Methods and Materials:

- Screening
  - Venous blood draw
  - MRI Screening Form

  - Medical History
  - Demographics
  - Medication Tracking Log
- Maximal Exercise Visit for cardiorespiratory testing
- Study visits:
  - IV catheter, blood sampling (if the blood sampling cannot be obtained or completed, it is not a requirement to continue with other study procedures).
  - MRI scan sequences (T1, ASL, and fMRI)
  - Oral pill(s): AOC or placebo. All other study visits are identical, apart from the study pill selection.
  - Hyperoxia (increasing SpO_2 to 100%, normal CO_2) – stressor used to change CBF, while bleeding in a small amount of CO_2 to keep $ETCO_2$ at normoxic levels.
  - fMRI tasks: verbal fluency and a rotating checkerboard task.

Subject participation: 3-4 lab visits:

1. Consent and screening visit
2. Maximal Exercise visit (cardiorespiratory fitness testing; can be combined with screening if eligible)
3. MRI visit (placebo or AOC)
4. MRI visit (placebo or AOC); visits 3 and 4 are randomized double-blind placebo controlled

2.2. Detailed Rationale

Cerebrovascular disease is the third leading killer in the U.S., which exhibits a dramatic sex-specific risk pattern. Men exhibit lower resting cerebral blood flow (CBF) and two-fold greater risk of cerebrovascular disease compared to premenopausal women. Despite this sex-specific disease risk, the essential mechanisms controlling CBF in humans are not well-defined. A lack of physiologic insight is highlighted by the controversies in optimal sex hormone treatments—and adverse health outcomes—seen in important clinical populations including aging, PCOS, and gender reassignment. Herein we aim to address the gaps in knowledge of human cerebrovascular physiology prior to confounding factors like age and disease, in order to identify sex differences and similarities in how CBF is controlled.

This proposal addresses these limitations in healthy men and women. First, we leverage the state-of-the-art MRI technique called Arterial Spin Labeling (ASL). This MRI approach quantifies CBF responses in microvascular perfusion of discrete brain regions at rest and during hyperoxic stress. Importantly, we test mechanistic control of ROS in hyperoxic responses by acute treatment with AOC.

Brain function critically depends on a close matching between metabolic demands, appropriate delivery of oxygen and nutrients, and removal of cellular waste. Neurovascular coupling serves as a crucial mechanism that ensures a rapid increase in the rate of CBF and oxygen delivery to activated brain structures. Disruption of CBF regulation may represent a major factor in the development and progression of Alzheimer's disease and other neurodegenerative diseases.

It's been shown that lower levels of CBF in Alzheimer's disease may reflect synaptic failure (Chen, Rosas, & Salat, 2011; Musiek, Chen, Korczykowski, Saboury, Martinez, Reddin, Alavi, Kimberg, Wolk, Julin, Newberg, Arnold, & Detre, 2012), resulting in reduced metabolism and reduced demand for CBF (Iturria-Medina, Sotero, Toussaint, Mateos-Perez, & Evans, 2016; Iadecola, & Gottesman, 2018). This synaptic failure then continues throughout the course of the disease and is associated with the cognitive decline in the later stages (Landau, Mintun, Joshi, Koeppe, Petersen, Aisen, Weiner, & Jagust, 2012). In this scenario, reduced CBF is not a causal factor in neurodegeneration but a consequence of loss of synaptic

function. Support for this notion comes from recent studies that reported that cerebral global hypoperfusion in the general population is related to accelerated cognitive decline (Wolters, Zonneveld, Hofman, Van Der Lugt, Koudstaal, Vernooij, & Ikram, 2017; Yew, & Nation, 2001-2017) and increased risk for dementia (Wolters et al., 2017).

Evidence demonstrates that brain activity, particularly synaptic function, can be evaluated using BOLD fMRI data. Functional tasks like verbal fluency tests or checkerboards can significantly impact this activity and serve as effective neuropsychological assessments for dementia detection and severity measures (Greicius, Krasnow, Reiss, & Menon, 2003; Raichle, MacLeod, Snyder, Powers, Gusnard et al., 2001; McKiernan, D'Angelo, Kaufman, & Binder, 2006; Kelly, Uddin, Biswal, Castellanos, & Milham, 2008). We believe that BOLD fMRI offers a noninvasive approach to quantify CBF and has seen growing utilization for visualizing brain functions by assessing either resting CBF or its responses to physiological stressors.

We are interested in the investigation of the impact of hyperoxia-induced reductions in CBF on brain activity during cognitive tasks. Specifically, we seek to determine the extent of variation in brain activity between room air conditions and hyperoxia while participants engage in cognitive testing.

2.3. Innovation

Our preliminary data clearly demonstrate sex differences in CBF control to hyperoxia that are specific to brain regions. Data generated in these studies will set the stage for designing mechanistic, sex-specific studies in focused disease populations who exhibit sex-specific etiology (e.g. diabetes, hypertension).

Regionally specific: Brain pathology is often region-specific. Analyzing the entire circulation allows us to test sex-specific cerebrovascular pathophysiology that may exhibit regional specificity.

Quantifiable: ASL quantifies CBF with reasonable temporal and high spatial resolution.

Expected Findings: These studies will generate an important breadth and depth of new understanding of sex-specific CBF regulation because these data: 1) are obtained *in vivo* in humans, 2) explore fundamental sex differences in stress responses, 3) quantify sex-specific and region-specific differences in mechanistic control, 4) demonstrate sex-specific CBF control mechanisms are established in young adults *without* cardiometabolic or aging risk factors.

3. SPECIFIC AIMS AND PRELIMINARY EVIDENCE

In previous IRB approved studies, we established an experimental setup where participants can be scanned with a head coil while wearing a mask to control inhaled gases in the MRI. CBF is measured in normoxic rest and during a steady-state hyperoxic challenge, with the ASL technique. Hyperoxia increases arterial O_2 (S_aO_2) to 100% while maintaining CO_2 at normoxic levels. Importantly, hyperoxia falls under the scope of the grant to study stressor responses in CBF regulation.

Data indicate CBF declines by ~20% in response to hyperoxia, which is similar between sexes. However, several regions indicate they do not decrease CBF to the same extent, and some regions show sex differences in hyperoxic responses. Data from a small group of healthy males (Mattos et al., 2019) indicated the CBF reductions can be attenuated with antioxidant treatment (Mattos et al., 2019). A clear limitation is the lack of females in that study. Second, CBF was measured with Doppler ultrasound at the neck, which precludes any insight into brain regional analysis of hyperoxic responses. Third, to our knowledge only one study has looked at durations of hyperxia longer than 10-15 minutes of hyperoxia

exposure (Damato et al., 2020); our protocol will extend for 40 minutes to address how stable the reduction in CBF is beyond 15 minutes.

3.1 Specific Aim

Test the hypothesis there are sex differences in hyperoxic vasoconstriction, due in part to ROS signaling.

Aim 1A: Hyperoxic reductions in CBF will be greater in males than females.
Aim 1B: Sex differences may present regionally specific patterns (non-uniform) by sex.
Aim 2: Acute antioxidant treatment to reduce ROS will limit hyperoxic reductions in CBF more in males than females.

3.1.1. Rationale

While several studies investigated CBF responses to hyperoxia, none have addressed potential sex differences. These data could have important clinical or military applications in a sex-dependent manner. Second, MattosClick or tap here to enter text. showed scavenging ROS with vitamin C infusion attenuated hyperoxic reductions in CBF, indicating ROS contribute to hyperoxic vasoconstriction (Mattos et al., 2019). A limitation of this study is that it was only performed on males.

3.1.2. Preliminary Data

The feasibility for our Aims is supported by our successful preliminary studies completed under IRB 2020-0336 (the parent R01 supporting this and other protocols). In 8 males and 7 females, CBF reductions tended to be greater in males. Related to the current design using AOC, our lab has scavenged ROS in past to manipulate vascular function, which can change vascular responses to various pharmacologic or physiologic stressors.

3.2. Expected Results

We predict the overall reduction in CBF will be greater in males than females, but that some region by sex differences will be apparent. We also predict antioxidants will limit the CBF reductions, and this effect will be more pronounced in males.

3.3. Study Duration

Expected start date 07/15/2023
Expected end date: 05/30/2025

Subjects can expect their participation to last in this study anywhere from one week to six months. The estimated length of participation varies due to several factors, including: subject availability, MRI availability and timing of menstrual cycle for female subjects.

4. RESEARCH DESIGN AND METHODS

4.1. Subject Population

Phase 3: Thirty (30) otherwise healthy adults between 18-40 years of age composed of 15 males and 15 females. These subjects will use similar inclusion/exclusion criteria as our two earlier protocols under this NIH grant (2020-0336 and 2023-0548), such that some recruitment can occur from subjects who enrolled in those protocols. However, we will recruit and screen interested individuals who did not

participate in the previous phases of the study, using standard Schrage lab recruiting and screening approaches (ex. mass email, fliers, or advertisements) as well. There is no minimum or maximum time that can occur between participation of this phase of the study and the previous phases.

4.1.1. Inclusion Criteria

Subject will be included if they meet all the following criteria:

Age between ≥18 - ≤40 years

4.1.2. Exclusion Criteria

Subject will be excluded if they meet any of the following criteria:

- Hypertensive
  - >125 mmHg systolic blood pressure; or
  - >80 mmHg diastolic blood pressure
- BMI ≥25 kg/m^2
- Fasting blood glucose ≥100 mg/dl
- LDL cholesterol ≥130 mg/dl
- Triglycerides ≥150 mg/dl
- Current diagnosis or history of:
  - peripheral vascular disease
  - hepatic disease
  - renal disease
  - lung disease
  - gastrointestinal disorders/bleeding
  - hematologic disease
  - stroke
  - myocardial infarction
  - coronary heart disease
  - congestive heart failure
  - heart surgery
  - prediabetes
  - diabetes mellitus (type 1, type 2, MODY, or others)
  - sleep apnea
  - hypertension
  - some autoimmune diseases, such as inflammatory bowel disease or systemic lupus erythematosus (exclusion at discretion of reviewing MD)
- Current smoking, defined as the use of tobacco or nicotine products >5 times in the past 30 days.
- Cardiovascular medication use
- Taking NSAIDs prescribed by a physician for clinical care
- Any contraindications of having an MRI
  - (e.g. the requirement of anxiolytics in order to complete an MRI scan)
- Irregular menstrual cycle (females only)
- Medical conditions that can affect the menstrual cycle, such as hyperprolactinemia, prolactinoma, hypercortisolemia, and congenital adrenal hyperplasia (females only)
- Pregnancy, breast feeding, or plans to conceive within the next 3 months (females only)
- Polycystic ovary syndrome (females only)
- Hirsutism defined as unwanted and/or excessive hair growth on the face, chest, or back (females only)

- Levonorgestrel intrauterine device (IUD) (females only)
- Hormonal birth control will not be allowed in women, in order to control for high variability between type, dose, and route of therapy. However, in discussion with Dr. Davis (Co-I) and Dr. Laura Cooney M.D., our physician experts in medical and reproductive endocrinology and infertility, there are two broad exceptions to this birth control criteria:
  1. Copper intrauterine devices (IUDs) will be allowed as they do not change systemic sex hormone levels).
  2. Women currently taking hormonal birth control (i.e. contraceptive pills, patch, ring) for contraception only (not for a medical condition such as those listed in exclusion criteria above) may consider temporarily stopping to become eligible for enrollment. Hormonal birth control must be stopped at least one month prior to Study Visit 1 to provide time for menstruation to resume. Then stoppage continues through the last planned study visit. Screening information will be reviewed by our endocrinology physicians to determine eligibility and timing on this issue (details below).

Eligibility Determination Clarification: At each screening visit we take two separate readings, from the same blood draw, of each of the following blood values: glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and hemoglobin. When determining eligibility, the study team member (graduate student, staff, post-doc, P.I. or MD) will use the average of these two values to determine the subject's eligibility. This average value will be the value we use for eligibility even if one of the values from the two initial readings exceeds the eligibility limit.

4.1.3. Subject Identification and Recruitment

30 participants (15 per biologic sex) will be identified and recruited from participants who have completed our prior MRI studies noted above or the general population. We will keep enrollment open until we reach our goal of 30 participants completing all assigned visits.

Subjects from the general population will be recruited through the University's email of faculty, staff, and students. This email recruitment service will be provided by the Division of Information Technology (DoIT) and will be sent out 2-6 times per year depending on recruitment success.
All recruitment procedures will be reviewed and approved by the UW IRB.

Subjects who indicate on the 2020-0336 Informed Consent form that they are interested and willing to participate in other lab studies will be contacted by email a maximum of 5 times to schedule a time to come in for the Phase 2 screening visit to determine eligibility. If the subject does not answer after 5 contact attempts, they will be removed from the email list.

4.1.4 Withdrawal of Participants

Participants may be withdrawn from the research for the following reasons:

- The subject's health changes and participation in the study is no longer in their best interest
- The subject does not follow the study rules
- The subject no longer meets the requirements to be in the study
- The study is stopped by the sponsor or researchers

4.2. Research Sites

The following sites will be used for the following purposes:

- Medical Sciences Center
  - Consent
  - Screening questionnaires
  - Maximal Exercise Test
- UW Human Exercise Research Core (HERC) in the UW Nursing School
  - Maximal Exercise Test
  - Consent
  - Screening questionnaires
- Wisconsin Institute of Medical Research (WIMR)
  - MRI/fMRI
  - IV placement
  - Consent
  - Screening questionnaires
- Waisman Center
  - MRI/fMRI
  - IV placement
  - Consent
  - Screening questionnaires

Consenting at the research sites of WIMR and Waisman are to allow for re-consent of subjects, when that process is necessary. This also allows for subjects participating in other phases of the study (IRBs: 2020-0336 and 2023-0548) to provide consent for participation in this study at their study visits. This flexibility to re-consent, consent, and screen for this study at these sites (during participation in another phase of the study) eases the burden on both the subject and study team, so they do not have to schedule another appointment at the Schrage lab to conduct these procedures. The same informed consent process will be conducted at all sites.

4.3. Experimental Procedures

4.3.1. Pre-Screening Subjects

Subjects who complete our prior MRI studies under this grant (2020-0336 and 2023-0548) will be invited back into the laboratory for a more in-depth in-person screen (if outside 180 days from their most recent screen).

For new subjects recruited from the general population, determination of eligibility will be a two-step process. Subjects will be initially screened via a REDCap survey, which will be found through a link/QR code in the recruitment material. If eligible based off of this survey, they will be invited in for a more in-depth in-person screen. Consent/permission will be obtained for pre-screening following a description of the study and prior to survey questions either:
• Verbally for phone screen
• Agreeing to the REDCap survey

The same script will be used for both the phone screening and REDCap survey (i.e. the REDCap survey will be discussed with the subject verbally and constitutes the “phone screen”).

Delegated study team members will screen patients for eligibility for the study based on medical history, medication use, and evaluate general safety to undergo MRI (e.g. claustrophobia, metallic implants, etc.).

These steps will be taken to minimize risk of participation. If an individual does not preliminarily meet all inclusion criteria or preliminarily meets any of the exclusion criteria, then the subject will be immediately eliminated from consideration for the study and their information will be destroyed (e.g. shredder or digital deletion). If the subject reapplies for the study due to a change in weight, medical history, or health status that has previously made them ineligible they are able to be re-screened for eligibility.

4.3.2. In-Person Screening

Duration: Approximately 1 hour

A screening visit reminder will be provided to potential subjects via phone or email at least 12 hours prior reminding them of the following.

- Location of visit (with general campus directions)
- Fasting directions

Subjects will be given:

- Signed copy of the informed consent
- Information sheet

After informed consent is obtained, the following information will be collected during the screening visit:

- Health history questionnaire
- MRI screening questionnaire
- Demographics
- Medication Tracking Log
- Female participants must first take a urine pregnancy test (commercial test provided by study team) to rule out pregnancy
- Height & weight
- Hip & waist circumference
- Resting blood pressure
- Venipuncture blood sample
    - Glucose, lipid panel to test for inclusion/exclusion criteria

Blood pressure, questionnaires, and venous blood sample will be used at screening to further determine eligibility.

All blood measurements taken during the screening procedures will be analyzed by Schrage lab staff.

The results of the screening procedure will be valid for study inclusion for 180 days. If the subject does not enroll in the study (actively start study visits), he/she must undergo screening procedures to reassess eligibility. The eligibility criteria and screening content is identical to two other protocols under this NIH funding (2020-0336 and 2023-0548). We will deem the subject eligible for this protocol if the prior screening visit occurs within 180 days.

4.3.3. Exercise Test (if subject falls out of the 180-day window of other protocols)

Duration: Approximately 1 hour

Subject meets staff at HERC in nursing school. Subject does not need to be 8 hours fasted for these procedures. Maximal exercise test is used to determine cardiorespiratory fitness. The treadmill test usually takes 8-12 minutes. The tests starts at 5mph and 3% incline and increases 1mph and 1% incline every 2 mins. Subject is monitored for HR and oxygen consumption (VO_2) in an incremental test to volitional exhaustion.

4.3.4. Study Visits

Duration: Approximately 2 hours each (60 min for study checklist, IV insertion 45 min prior to MRI scan, 61-minute MRI scan)

A visit reminder will be provided to potential subjects via phone or email at least 12 hours prior reminding them of the following:

- Location of visit
- Fasting directions
- Caffeine, NSAIDS, and exercise restriction

Due to the unpredictability of the menstrual cycle and MRI availability, if an MRI timeslot becomes available, we will confirm with the subject that they meet the fasting and restriction directions listed above and schedule them for the MRI timeslot. On the day of the visit, we will confirm that the subjects have followed the protocol.

The mean half-life of antioxidants is estimated to be 2-4 hours (Padayatty et al., 2004). Due to the short half-life, MRI visits can be scheduled to occur at a minimum of 24 hours apart for the same subject. This timing is crucial to keep scientific integrity high (for placebo/AOC effects) while optimizing matching of subject availability with MRI scheduling constraints.

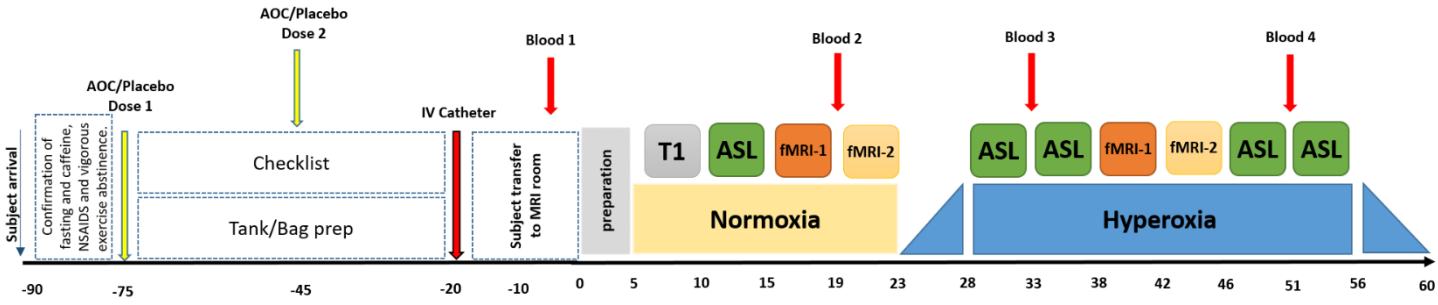
Study visits will be performed at the WIMR imaging facility (1111 Highland Ave., Madison, WI), or Waisman Center (1500 Highland Ave., Madison, WI) and experience the following procedures:

- A study team member will verbally review with the subject:
    - Concomitant medication
    - Has fasted for a minimum of 8 hours prior to data collection for study visit (MRI scan)
    - Abstained from vigorous exercise and NSAIDs medication ≥24 hours prior to study visit
    - Abstained from caffeine ≥24 hours prior to study visit
- Female participants must first take a urine pregnancy test (commercial test provided by study team) to rule out pregnancy. Study staff will determine and record the result on study form.
- A study staff member will complete and review an MRI Safety screening with the subject.
- IV catheter and blood sampling: An intravenous (IV) catheter will be placed by qualified staff. Research laboratories will conduct the blood testing noted below. Blood will be sampled for markers of oxidative stress/ROS as well as markers of efficacy of antioxidant cocktail (vitamin C levels, MDA) during normoxia and hyperoxia. The blood draws during the study are an optional portion of the study visit, the study may still occur if the study team is unable to obtain the IV placement or the draws themselves. Whether the blood draws occur at the study visit will be determined by the members of the study team.The researchers may acquire all the draws, some or none during a single study visit; this all is on depending timing, subject willingness, blood flow or other inhibiting factors. These blood draws generally occur: 1) prior to entering the MRI, 2) during Normoxia, 3) near the beginning of Hyperoxia, and 4) near the end of Hyperoxia.

- Prior to the study visit, subjects will be randomized into the placebo or AOC protocols. Randomization will consist of a study member utilizing a random number generator to sort subjects into placebo (even number) or AOC (odd number).
- Subjects will arrive 120-90 minutes prior to MRI scanning to take Dose 1 of either the AOC or placebo, and then Dose 2 60 minutes before MRI scanning begins.
- For the AOC:
    - Dose 1- 300 mg α-lipoic acid, 500 mg vitamin C, 200 IU vitamin E
    - Dose 2- 300 mg α-lipoic acid, 500 mg vitamin C, 400 IU vitamin E

4.3.4.a. Visit A and Visit B Protocols (Randomized - AOC/Placebo)

Figure 1: Timeline for MRI Visits. Two visits are identical with the exception of Placebo vs AOC treatment (randomized)



- **Hyperoxia (High Oxygen):** An increase in blood O_2 is a powerful signal to decrease cerebral blood flow. Subjects will breathe in air that has increased levels of oxygen (100% O_2). Subjects will breathe this air for ~30-45 minutes. Once the trial is over, subjects will be switched back to breathe room air and remain in the scanner while they return to baseline values.
- After baseline structural/localization scans, MRI ASL scans will be completed at the following conditions:
    - Normoxia baseline
    - Steady-state hyperoxia

4.3.5. Details of Procedures

4.3.5.a. Informed Consent

Due to the screening visit requiring a fast of ≥8 hours an alteration of consent process and a waiver of documentation is needed.
Alteration of Consent Process: This research involves no more than minimal risk to the subjects. The alteration does not adversely affect the rights and welfare of the subjects. The screening visit could not practically be carried out without the alteration. Whenever appropriate, the subjects will be provided with additional pertinent information after participation. Fasting for 8 hours prior to the screening visit is needed to determine the fasting glucose and lipid levels of the subjects. This allows the research team to determine eligibility of the subjects for the study.
Waiver of Written Documentation of Consent: Written information to be provided includes all required and appropriate additional elements of consent disclosure. The task of fasting is no more than minimal risk of harm to subjects. Fasting is a procedure for which written consent is normally not required

outside of the research context.Written information describing the research is to be provided to the subject.

Informed consent/assent will be obtained from each participant outlining the potential risks. Consent will be obtained at the initial screening visit and will take place prior to any other procedures being performed. If written consent/assent is refused, the subject will no longer be considered for the study. Schrage lab personnel will follow HRP-090- SOP – Informed Consent Process for Research and HRP-091- SOP – Written Documentation of Consent.

1. Schrage lab personnel delegated to obtain consent discusses the study in detail with the potential research subject, covering each of the required elements of the informed consent process.
2. The consent process will originally take place at Dr. Schrage's lab space in the Medical Sciences Center. However, if reconsent is needed, it may take place at Medical Sciences Center, WIMR, Waisman, UW HERC in the UW Nursing School, or UW Hospital Department of Pediatrics.
3. The research subject will be given a copy of the informed consent document and the consent information sheet to read in a quiet environment without distraction.
4. The research subject will be encouraged to take these forms home so that he or she may discuss it with family members, friends, clergy, or others when possible.
5. All questions and concerns will be addressed throughout this process by the person obtaining informed consent and/or PI.
6. If a research subject decides to participate, he/she will be asked to sign the most current IRB approved informed consent document only after all questions and concerns have been addressed and the person obtaining informed consent is satisfied that there is a clear understanding of the research study.
7. The Informed Consent Process Documentation Checklist will be completed.
8. If re-consent is required (which is checked prior to each study visit), the Informed Consent Process Documentation Checklist will be completed again.
9. The study team members will review the informed consent document to ensure that all fields that require a response are complete (i.e. checkbox marked yes or no, etc.).
10. The informed consent document must be signed and dated by the research subject and signed by the person obtaining informed consent.

4.3.5.b. Fasting, Caffeine, NSAID, and Exercise Restrictions

Prior to the in-person screening visit and each MRI Study Visit each subject will be asked to:

- fast for a minimum of 8 hours
- refrain from using NSAIDs (e.g. Ibuprofen) for a minimum of 24 hours
- refrain from caffeine for a minimum of 24 hours
- refrain from vigorous exercise for a minimum of 24 hours

4.3.5.c. MRI Screening Document

Each subject will be screened for MRI safety first during the pre-screening process. At the initial screening visit, the subject will fill out an MRI screening document provided by the research team to ensure their safety and comfort in the MRI scanner.

4.3.5.d. Maximal Exercise Test

Each subject will complete a maximal exercise test on a treadmill. Subjects will be fitted with a mask or mouthpiece to measure oxygen consumption (VO_2) and carbon dioxide production (VCO_2), and a Polar Heart Rate Monitor to measure heart rate. Following a 2-minute warm-up at 3.5 mph, exercise intensity increases in speed and grade every 2 minutes until the subject can no longer continue despite strong verbal encouragement. Average duration of maximal exercise testing is approximately 8-12 minutes (45, 48). If performed on different day than In-Person Screening Visit, the subject does not need to be 8 hours fasted.

4.3.5.e. Temporarily suspending hormonal birth control

Women who regularly take hormonal birth control (i.e. contraceptive pills, patch, and ring) for contraception will be asked if they are willing to temporarily stop taking birth control until their MRI study visits are completed. The study team will prioritize scheduling female subjects who temporarily stop taking birth control in order to minimize the duration of time that they will be off contraceptives. Women who take hormonal birth control for medical conditions, such as PCOS, prolactinoma, or very heavy menstrual bleeding will not be allowed in the study as their hormonal birth control should not be stopped. Screening will determine the type of birth control, which will be reviewed by our collaborating physicians specializing in medical and reproductive endocrinology. After the subjects consent to participate in the study, the study physicians will determine the appropriate number of days to withdraw birth control prior to MRI study visits. The resumption of menses as our primary marker that women have returned to “normal” hormone levels. However, to ensure scientific validity, blood samples will be taken on one of the MRI study visits to assess hormone levels. Once the woman completes the MRI visits, she can choose to resume birth control. Resumption of birth control will also be coordinated with the study physician to ensure it is done in an appropriate and safe manner.

4.3.5.f. Magnetic Resonance Imaging (MRI)

Images will be acquired on a GE 3T MRI system located in the WIMR within the UW Department of Radiology, or at the Waisman Center Brain Imaging Core. The research team will be administering the scans, as well as processing and analysis of the data. No contrast agent will be used. While in the scanner, subjects will be monitored for vital signs. All MR scanners currently used at the University of Wisconsin stay within FDA guidelines. All pulse sequences used in this study are designed to stay within the current guidelines for dB/dt established by the FDA.

The software and head coil used in this study are not intended for use in the diagnosis or treatment of subjects, nor are they being assessed for safety or efficacy as part of this study. Study data will not be submitted to the Food and Drug Administration (FDA) in support of labeling changes for these devices.

Our main outcome is focused on brain perfusion using Arterial Spin Labeling (ASL), so we can look at global and regional perfusion across time exposure of hyperoxia. This method is well-characterized and used routinely during collaborative studies between Drs. Wieben and Schrage.

Also, fMRI scans will be collected while the subject is performing tasks in the scanner to identify the brain regions engaged during task performance and to assess how these responses correlate with measures of CBF and other perfusion metrics.

fMRI tasks will include a verbal fluency task and a rotating checkerboard task. The verbal fluency task involves word retrieval, which is used as a measure of executive function. Specifically, this involves

presenting a letter to the subject and asking them to covertly generate words starting with that letter. 20-second blocks of task are interspersed with 20-second blocks of rest. The visual task involves the subject looking at a green dot in the center of a rotating checkerboard. Instructions to start and stop task performance are communicated to the subject using the communication system already present in the scanner. Other equipment, such as the visual goggles or auditory equipment for delivering visual and auditory stimulus or motor response device for collecting subject responses, are under the control of the researcher or scanner operator and may be used as well.

Participants will undergo the fMRI tasks twice: once under normoxia and once under hyperoxia, with each session lasting approximately 4-5 minutes for a total of 8-10 minutes. The fMRI scans will be conducted between ASL scans.

Waisman Center only: Application of Investigational Hardware and Software: Any or all of the pulse sequences (scanner software) may be custom pulse sequences developed by Dr. Alexander's lab or project collaborators. These custom research pulse sequences are not FDA approved; however, they are adapted from FDA approved GE product pulse sequences and follow all of the regulatory safety requirements for RF (radiofrequency) power and magnetic field switching (dB/dt). Custom research pulse sequences are commonly performed in MRI research using the EPIC pulse sequence software development tools from General Electric for research use on their MRI scanners. The safe use of these MRI pulse sequences is outlined in a letter from Andrew Alexander (as Waisman Brain Imaging Lab Core Co-Director). Image data acquisition may be performed using a 32-channel receive-only head coil from Nova Medical (Boston). The Nova coil is not an FDA approved device, but it was developed for safe use in humans and follows all of the regulatory requirements for human use. This coil is now widely used in neuroimaging research studies at the Waisman Center. As both the investigational hardware and software will operate within FDA guidelines, the MRI studies are considered nonsignificant risk. These technologies are not being evaluated for performance or safety in this study.

4.3.5.g. Venipuncture

Occurs only during the screening visit. Venipuncture will usually occur in the antecubital fossa or hand by trained personnel using standard aseptic technique. If staff fail to obtain blood during screening, we will ask the participant to return to lab another day to attempt a new fasted blood draw.

4.3.5.h. Intravenous Catheter

An IV catheter will be placed by trained personnel in the antecubital fossa or hand using standard aseptic technique. WIMR staff, research nurse, phlebotomy trained individuals, or higher study-associated clinical authority (e.g. physician) will insert an IV using standard medical procedures. The IV catheter will remain in place for up to 2 hours. If the blood sampling cannot be obtained or completed, it is not a requirement to continue with other study procedures.

4.3.5.i. Blood Sampling

Screening visit:
Up to 20 mL of blood will be obtained for blood chemistry values to determine eligibility.

IV catheter on both MRI study visit days:

Blood draws will be obtained via IV catheter. The volume taken on study day is expected to total ~120 mL before entering the MRI and ~10mL over 3 time points in the MRI. The specific time points are as follows:

1. Before entering MRI (for sex hormone levels [one of the two visits], and blood markers of ROS/antioxidant activity)
2. During normoxia baseline (to confirm sample 1 above for stability)
3. 5-10 mins into steady-state hyperoxia exposure (blood markers of ROS/antioxidant activity)
4. 30-45 minutes steady-state hyperoxia, (blood markers of ROS/antioxidant activity)
5. If the blood sampling cannot be obtained or completed, it is not a requirement to continue with other study procedures.

4.3.5.j. Hyperoxia

Subjects will breathe room air quietly while lying in the MRI. After baseline data collection, subjects will breathe a gas mixture of 100% O_2. We will also bleed in CO_2 so subjects maintain a constant end-tidal CO_2 (inspired CO_2 will be between 1-5% of inspired air). Arterial oxygen saturation will be measured by finger pulse oximeter. Subjects will breathe this gas for 30-45 minutes. We will measure the rise in end-tidal CO_2 with an MRI compatible end-tidal CO_2 analyzer. After 30-45 minutes of hyperoxia gas exposure, subjects will return to breathing room air, and we monitor them to ensure all variables return to baseline levels (typically within 1 minute). The study team will be purchasing medical grade oxygen and gas tanks will be supplied by Airgas. When not in use, gas tanks will be kept in a secure tank storage room connected to the MRI suite which only research staff have access to.

4.3.5.k. Randomized Drug Supplementation

Antioxidant pills
Previous cardiovascular antioxidant research using either acute or chronic dosing led us to choose the following total dosing of 1000 mg vitamin C, 600 IU vitamin E, and 600 mg of alpha lipoic acid (Bunsawat et al., 2020; Wray et al., 2012; Wray, Uberoi, Lawrenson, Bailey, & Richardson, 2009). The first dose will be 500 mg vitamin C, 200 IU vitamin E, and 300 mg of alpha lipoic acid, and will be orally ingested 120-90 minutes prior to MRI scanning. The second dose will be 500 mg vitamin C, 400 IU vitamin E, and 300 mg of alpha lipoic acid, and will be orally ingested 60 minutes prior to MRI scanning. This dosing scheme significantly reduces blood markers of ROS while increasing markers of blood antioxidant capacity. Subjects will be randomly assigned in a double-blinded manor to receive either the antioxidant pills or placebo pills for each MRI visit. If the subject is randomly assigned to receive the antioxidants for Visit A, they will then receive the placebo pills for Visit B and vice versa. In consultation with the FDA, it was determined that these antioxidant pills are classified as a dietary supplement and, therefore, do not require an IND.

Placebo:
Placebo pills of similar color, shape and size to antioxidant pills will be given to subject for the placebo visit. These are most likely lactose or microcrystalline cellulose pills made for human ingestion.

4.3.5.l. Subject Monitoring

Throughout the MRI visits, subjects will be monitored for:

- Heart rate (via electrocardiogram or pulse oximeter)
- Blood pressure (via automated brachial artery auscultation)
- Blood oxygen saturation (via pulse oximeter)
- End-tidal carbon dioxide

- The frequency of monitoring is as follows: during baseline and each major MRI sequence (typically every 5-7 minutes). Visually, HR, SpO_2 and $ETCO_2$ are continuous readouts on patient monitor. Practically speaking, all are recorded every 5-10 minutes on CRFs. These are monitored by trained laboratory staff who are physiologists-typically graduate students, postdoctoral fellows, or PI who are knowledgeable about stopping guidelines. All recorded values are on CRFs with clearly stated stopping guidelines.

For all devices used to monitor this study (pulse oximeter via patient monitor, automated brachial artery auscultation), these devices are not being evaluated for safety/efficacy and are being used within their FDA indications as tools to achieve the aims of this research.

After each MRI visit subjects will be contacted ~24 hours later. A study team member will try to contact them up to 3 separate times.

4.4. Remuneration

Subjects will be paid in the following manner:

Visit or procedure	**Payment amount**
Screening Visit	$20
Exercise Visit	$20
MRI study visits	$30/hour; rounded to the nearest half hour
Completing both MRI study visits	Bonus $30
If you complete all procedures and visits	~$190-220 total

Payment will be provided at the end of the study visits. If participants choose to leave early or are taken off study for any reason, payment will be pro-rated. If any study visits are repeated for any reason (fitness test, gas challenge, failed blood draw, stopped MRI scan due to technical/scheduling errors), participants will be paid for additional study time based on rates above.

5. BANKING AND SHARING DATA

Blood analysis results and MRI images will be stored for future research use. Data/specimen banking is not optional for participants. Studies utilizing banked data and/or biospecimens will be approved by the IRB. Banked data from blood analyses and MRI images will be stored on secure computer servers that are password-protected. Any data shared with personnel outside of the study team will be coded with a study ID number. Unless the staff is listed as key personnel on this study, they will not be given access to the key linking subject identifiers with their data or study ID number. The study PI will be responsible for the oversight of the data banking and will review all requests to utilize the data and images. The PI will be responsible for confirming that IRB approval or exemption has been granted prior to the release of any data. Future study results obtained from banked data will not be reported to subjects. For subjects to withdraw their permission to bank data/specimens they will need to send Dr. Schrage a letter indicating their reason for withdrawal, then data/specimens will be destroyed and deleted from storage. Most of the tests that are part of this study are for research purposes only. Because of this, we will not tell the subject or their doctors the results of these research tests.

6. RISKS ASSOCIATED WITH PROCEDURES

6.1. Risk Summary

This protocol is minimally invasive with IV catheter for blood sampling. Over the counter antioxidant supplements are considered very safe. MRI sequences are very safe. Short term gas challenges hyperoxia are well-tolerated, and easily reversible. Subject monitoring during study visit allows acute identification of AEs and subsequent action.

6.2. Detailed Risks

6.2.1. Fasting, and Caffeine, NSAID and Exercise Restriction

Risks include:

- feelings of hunger
- irritability
- fatigue
- light-headedness
- dizziness

There are no risks of NSAID restriction if NSAIDs are not prescribed by a physician for clinical care. If subjects are prescribed NSAIDs by a physician for clinical care, they will not be included in the study. There are no risks of acute abstention from exercise. These risks are considered minimal.

6.2.2. Intravenous Catheter and Venipuncture

Risks include:

- fainting
- bruise or clot formation
- infection
- pain at the site of catheter insertion/venipuncture
- hematoma after withdrawal
- soreness over the site

These should all be transient and resolve after several days.

6.2.3. Blood sampling

Risks include:

- Fainting
- Pain at site of blood draw
- Bleeding
- Infection
- Dizziness (unlikely since supine)

Poses no further risk than IV catheterization.

6.2.4 Maximal Exercise Test

Risks include:

- Feelings of exertion, fatigue, and breathlessness
- Discomfort due to VO_2 monitoring equipment (i.e., mouthpiece or mask)
- Serious complications including orthopedic injury, myocardial infarction, arrhythmia, hemodynamic instability, and death are rare (Takken 2016; van Brussel 2019)

These risks are considered minimal given the healthy population and age range, feelings of exertion, etc resolve quickly (seconds to minutes) after test termination.

6.2.5. MRI/fMRI

MRI uses a powerful magnetic field, not ionizing radiation, to create an image of the scanned area of the body. No contrast agent will be used.

Risks include:

- There are no known risks of MRI, aside from the standard risks associated with persons with certain metallic implants. If potential participants have a metallic implant, cardiac pacemaker, or are pregnant, they will be disqualified.
- Sensation of claustrophobia or anxiety, particularly in individuals susceptible to it.
- People with metallic implants, such as prostheses or aneurysm clips, or persons with electronic implants, such as cardiac pacemakers, the magnetic field generated by the magnetic resonance machine can cause a displacement or malfunctioning of these devices.
- We know of no risks or adverse effects from the radio signals used in this study.
- Some people have also reported tingling or tapping sensations, or muscle twitches in different parts of their body during the imaging procedure.
    - These sensations are not hazardous and should not cause discomfort to the participants.
- A small increase in risk may be associated with rapid gradient waveform switching times associated with fast MR imaging. In certain situations, the rapid switching of gradient waveforms has caused peripheral nerve stimulation in subjects.
    - Significant nerve stimulation, however, has not occurred as long as the imaging system has been programmed to stay within certain limitations of gradient strength and switching time (dB/dt).
- Occasionally, people who have clasped their hands tightly together during the study have reported a feeling of electrical shock in their hands and arms.
    - This is also not hazardous; however, to avoid any possible discomfort, participants will be instructed to not clasp their hands together during the study.
- Women who are pregnant must not participate in this study. The potential risks to a fetus from the MRI scan are not definitely known.
- The MR scanner produces loud tapping sounds during operation, which may reach somewhat objectionable levels.
- fMRI cognitive tasks performed in the scanner by the subjects, could cause discomfort due to the goggles required to be worn by the subjects. Performing the cognitive task themselves could led to subject fatigue.
- Anatomical abnormalities
    - Images will not be reviewed by neuroradiology
    - Any obvious abnormalities will be reported to the IRB upon discovery for assistance in determining how to proceed with potential reporting
        - A new information report will be filed with the IRB. This includes:
            - Description of the incidental finding
            - When it was incidental finding discovered

- Who discovered the incidental finding
- Rationale for disclosing the incidental finding (i.e., why the subject may benefit from knowing this information)
- Process for informing the subject of the incidental finding (i.e., who is going to disclose the finding, how, and who should the subject contact regarding questions)
    - Statement clarifying whether this is expected to occur again or if this is an isolated incident.

- Once the IRB approves informing the subject of the incidental finding the following will occur:
    - Our study physician (Dr. Eldridge) will contact the subject via email/phone to set up a time to discuss the finding.
    - The subject will contact Dr. Eldridge with further questions regarding the incidental finding.

6.2.6. Subject Monitoring

An automatic blood pressure cuff around the upper arm may feel uncomfortable while inflated, but this is temporary (30-60 seconds). Blood pressure measures are considered very safe.

6.2.7. Hyperoxia

Total exposure time to hyperoxia will typically last 30-45 minutes. There are two types of oxygen toxicity that are considered as risks, the Lorraine Smith effect which affects the pulmonary system and the Paul Bert effect which affects the central nervous system. Hyperoxia may decrease blood flow, increase oxygen levels, and has no known risks during the brief period (45 minutes or less) used here (Frank 1980). Risks of oxygen toxicity, which could damage lungs or nervous system, are a concern for those breathing increased concentrations of oxygen for extended periods, including underwater divers and those undergoing hyperbaric oxygen therapy. Occurrences of oxygen toxicity are rare in these conditions and therefore extremely low risk in the present situation. If this procedure makes subjects feel too uncomfortable, they can stop at any time.

The drug or biologic is lawfully marketed in the United States. The research is not intended to be reported to the FDA as a well-controlled study in support of a new indication for use nor intended to be used to support any other significant change in the labeling for the drug. The research is not intended to support a significant change in the advertising for the product. The research does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product. The research is conducted in compliance with the marketing limitations described in 21 CFR §312.7.

6.2.8. Antioxidant Treatment

All AOC pills used in this protocol are sold over the counter in the US and are food-grade. Side effects of these supplements are rare, but may include diarrhea, nausea, vomiting, abdominal cramps/pain, heartburn, tiredness, dizziness, or headache. When these occur, however, it is typically due to chronic dosing. With our study, subjects will only take these supplements acutely on one of the study visits.

6.2.9. Temporarily stopping birth control in women.

The largest risk of stopping birth control is pregnancy in sexually active women. We will reduce this risk by counseling patients to use a non-hormonal method (abstinence, condoms) while they are off birth control. The study team will prioritize scheduling female subjects who temporarily stop taking birth control in order to minimize the duration of time that they will be off contraceptives.

Additional risks of stopping hormonal birth control may include heavier, more painful, or more irregular periods and worsening of menstrual symptoms such as bloating, mood swings, or headaches. We will discuss these possible risks with subjects prior to their decision to discontinue hormonal birth control. Typically, if a woman did not have these types of menstrual symptoms before using hormonal birth control, they will not experience these symptoms with discontinuation. We will also exclude women who are taking hormonal birth control for specific medical conditions (such as PCOS, hyperprolactinemia, or very heavy menstrual bleeding) to prevent any medical risks of discontinuation.

7. DATA AND SAFETY MONITORING PLAN

7.1. Data Monitoring Committee (DMC)

The ICTR DMC provides investigators with independent services to ensure appropriate measures are in place to promote subject safety, research integrity and compliance with federal regulations and local policies for individual clinical research protocols in need of DMC review as determined by the PI, the funding agency, the local Scientific Review Committee, or the local IRB, and for which no DMC exists. For these studies, the UW ICTR DMC will be the primary data and safety advisory group for the PI.

The DMC is supported in its mission of safety and compliance by experienced ICTR staff to provide administrative assistance, experienced members representing a diversity of backgrounds, skills and knowledge, and the use of REDCap which allows more efficient tracking of protocols and protocol subjects. In providing oversight for the conduct of this study, the ICTR DMC will meet on a biannual basis throughout the study. Additional meetings may be scheduled as determined by the DMC or as requested by the PI. The DMC members will review protocol-specific reports created by statisticians that serve a non-voting member role on the DMC using data pulled from the REDCap. These standard reports will include an overview of study objectives, a review of actual and projected accrual rates, an evaluation of patient demographics for balance of randomization (if applicable), and a summary of the number and seriousness of adverse events. An interim analysis of study results may be performed and source documents may be reviewed to allow the DMC to independently judge whether the overall integrity and conduct of the protocol remain acceptable based on data provided and reported by the PI. The DMC will make recommendations to the PI that could include actions of continuation, modification, suspension, or termination.

Data Type	Frequency of Review	Reviewers
Subject accrual (including compliance with protocol enrollment criteria)	Every 6 months	ICTR Data Monitoring Committee, PI
Subject demographics	Every 6 months	ICTR Data Monitoring Committee, PI
Status of all enrolled subjects as of date of reporting	Every 6 months	ICTR Data Monitoring Committee, PI
Primary outcome analysis	Every 6 months	ICTR Data Monitoring Committee
AEs and rates	Every 6 months	ICTR Data Monitoring Committee, PI

SAEs	Per occurrence	ICTR Data Monitoring Committee, PI

7.2. Adverse Event (AE) Reporting

This study will utilize the Common Terminology Criteria for Adverse Events (CTCAE) v5.0 for adverse event monitoring and reporting. This information can be downloaded from the CTEP home page (http://ctep.info.nih.gov/CTC4/ctc_ind_term.htm).

The severity of the event will be graded using the CTCAE. In addition, for comparison to the CTCAE, adverse events will be tabulated using a 3-level schema as defined below:

- Mild
  - Event may be noticeable to patient
  - Does not influence daily activities
  - Usually does not require intervention.
- Moderate
  - Event may be of sufficient severity to make patient uncomfortable
  - Performance of daily activities may be influenced
  - Intervention may be needed.
- Severe
  - Event may cause severe discomfort
  - Usually interferes with daily activities; patient may not be able to continue in the study
  - Treatment or other intervention usually needed.

Note the distinction between the severity and the seriousness of an AE. A severe event is not necessarily an SAE. For example, a headache may be severe (i.e. interferes significantly with patient's usual function) but would not be classified as serious unless it met one of the criteria for SAEs.

Next, it will be determined by the PI and study physician or clinical neuropsychologists whether the event is expected or unexpected and if the AE is related to the intervention or study procedures. With this information, it will be determined whether an AE should be reported as an expedited report in addition to submission via routine clinical data.

Expedited AE reporting may require e-mail notification, phone, or fax submission of a written report as directed below. All expedited AE reports will be submitted to the IRB of record, the UW Health Sciences Institutional Review Board (HS IRB) in the case of this study.

7.2.1. Assessment of Attribution

Attribution of AEs will be assessed in relation to the study procedures. When assessing whether an adverse event is related to a study procedure, the following attribution categories are utilized:

- Definitely Related
  - An AE categorized as definite is clearly related to study procedures. If the timing of the AE is definitely consistent with the exposure to the study related procedures and it is most likely that the AE was caused by the study procedures such as because a high occurrence of the AE was expected based on the study intervention materials. In this case, the PI may categorize the AE as definitely related.

- Probably Related
    - An AE that is likely related to study procedures. If the timing of the AE is consistent with the exposure to the study intervention and it is more likely that the AE was caused by the study procedures than not, the PI may categorize the AE as Probable.
- Possibly Related
    - An AE that may be related to study procedure. If the timing of the AE is reasonably consistent with the exposure to the study intervention, and there is another cause of the AE that could be equally likely, the PI may categorize the AE as Possible.
- Unlikely Related
    - An Unlikely AE is one that is doubtfully related to study procedures. The coincidence of the AE with the exposure of the investigational product or intervention should be assessed. An AE that continued while the intervention was interrupted or stopped, or if the AE resolved while the intervention continued, may be categorized as Unlikely. If there is another more likely cause of the AE, the PI may determine that the AE was unlikely related to the study intervention.
- Not Related/Unrelated
    - An Unrelated AE is one that is clearly NOT related to study procedures. An AE may be considered Unrelated if the subject did not receive the study intervention or if there is another obvious cause of the AE (for example, a car accident or other disease/condition).

7.3. Serious Adverse Event (SAE)

A serious adverse event (SAE) is an AE occurring during any phase of the study (i.e., screening, admission, treatment, or follow-up) that fulfils one or more of the following criteria:

- Results in death
- Is immediately life-threatening
- Requires in-patient hospitalization or prolongation of existing hospitalization (more than 24 hours)
- Results in persistent or significant disability or incapacity
- Is a congenital abnormality or birth defect
- Is an important medical event that may jeopardize the subject or may require medical intervention to prevent one of the outcomes listed above

7.4. Identifying, Reviewing, and Reporting Adverse Events and Unanticipated Problems to the IRB

Identifying

- To effectively identify all AEs (anticipated or unanticipated), Schrage lab staff will follow laboratory and protocol specific SOPs, complete AE reporting forms for each subject as they arise and maintain a database of all reported adverse events for each protocol by entering all AEs into REDCap within a timely manner. All stopping events or subject reported complaints/concerns will be documented via study notes during the study. Participants will be encouraged to contact us with any questions or concerns following the study visit.

Reviewing

- The PI, study physician, and study team will also review AEs during internal audits of study documents in a cumulative fashion to identify any trends of safety concerns (see section 7.8 Internal Audits). In the event of an SAE, the PI, study physician, DMC, and IRB will review the event as soon as possible.

Reporting

- All adverse events will be recorded in REDCap in a timely manner consistent with reporting requirements. Schrage lab staff will log all AEs in REDCap, and report AEs and unanticipated problems to the DMC and to the IRB.

7.5. Expedited Adverse Event Reporting Requirements

Serious Adverse Event— Reported Within 24 Hours

- Serious Adverse Events requiring expedited reporting within 24 hours will be reported to both the UW Health Sciences IRB and the ICTR DMC Manager within one working day as directed below. Confirmation that all appropriate parties were notified will be done at this time.
- This includes:
    - Events caused by or associated with study participation
    - Events that are unexpected and immediately life-threatening or severely debilitating to current subjects or others not participating in the study

Adverse Event – Reported within 14 Days

- Adverse Events requiring expedited reports in writing within 14 working days will be sent by the PI and study team.
- This includes:
    - Information that indicates a new or increased risk, or a new safety issue
    - Harm experienced by a subject or other individual, which in the opinion of the investigator are unexpected and probably related to the research procedures.
    - Noncompliance with the federal regulations governing human research or with the requirements or determinations of the IRB, or an allegation of such noncompliance.
    - Audit, inspection, or inquiry by a federal agency that result in reportable findings
    - Written reports of study monitors that result in reportable findings (e.g., noncompliance with the IRB approved protocol)
    - Failure to follow the protocol due to the action or inaction of the investigator or research staff. (see Guidance for What Does Not Require Reporting to the IRB)
    - Breach of confidentiality.
    - Incarceration of a subject in a study not approved by the IRB to involve prisoners.
    - Complaint of a subject that cannot be resolved by the research team.
    - Unexpected change in study status by the sponsor, investigator, or institution that appears to impact subject safety and/or the ability of the study to meet its original aims (e.g., halt to enrollment due to equipment malfunction or drug supply issues). NOTE: This does not include meeting enrollment goals earlier than planned or a temporary suspension due to a planned interim analysis.

7.6. Protocol Deviations

To effectively identify deviations from the IRB approved protocol, Schrage Lab staff will maintain protocol checklists that are designed to not only minimize protocol deviations but to identify deviations when they do occur. Additionally, Schrage Lab staff will conduct regular internal audits (see section 7.8 Internal Audits) to identify deviations that may have been overlooked. All deviations will be logged on a Deviation Tracking Log and a database of deviations will be maintained in REDCap. The PI will review deviations as they are identified, and oversight committees will be notified according to their guidelines. The study team will implement corrective action plans when appropriate to address deviations.

7.7. Internal Audits

Regular internal audits will be conducted to ensure compliance and safety. Internal audits will occur on a quarterly basis. These audits could consist of full or partial review of study records, depending on observed past compliance. Audits will be documented on Internal Compliance Review forms modified specifically for the study and will include review of the following:

- Personnel (e.g., ensure all personnel have the appropriate training)
- Documentation of IRB submissions and correspondence
- Informed Consent/HIPAA Authorization Forms
- Laboratory Aspects of the Trial
- Equipment Maintenance
- Communication and Correspondence with oversight committees
- Study Conduct
- Completion of Case Report Forms
- Adverse Events
- Protocol deviations

These internal audits will identify any unanticipated outcomes or trends, and Schrage Lab staff can then notify the IRB of any new, common, or unanticipated adverse events, if applicable.

The results from internal audits (e.g., noncompliance, adverse event trends) will be discussed during regular lab meetings where team members can establish plans to prevent future issues.

7.8. Minimizing Research-Associated Risk

General Safety Overview: Our study has relatively low risk because of the age of the subjects ($\geq$18-$\leq$40 years) the minimal invasive procedures, and safe record of the study drug (antioxidants) in research settings. Gas challenges are also common, safe, easily reversed, and well-tolerated.

The procedures with the most potential for more than minimal risk are the venipuncture and intravenous catheter, and MRI, but we minimize risks by employing:

- Rigorous screening to determine eligibility
- Rigorous screening of women taking hormonal birth control prior to temporary discontinuation with consultation of collaborating endocrinologists.
- Venipuncture or Intravenous catheterization by trained study staff
- Clear study visit stopping guidelines for gas challenge or drug induced changes in hemodynamics.

Risks of venipuncture, or placing a venous catheter include bruise or clot formation and infection. The IV will be removed after ~1.5-2 hours. Minor risks associated with this procedure would be pain at the site of catheter insertion, bruising after withdrawal, and soreness over the site. These should all be transient and resolve after several days.

Subjects will be asked to notify research staff in they experience any side effects of antioxidant ingestion. In the unlikely event of distress, the physician on-call in the medical facility or on-site nursing coverage will be called.

Exercise visit performed under the oversight of trained staff. Subjects will always be monitored by research staff throughout the study visits.

Safety equipment:

MRI facility: Full-time staff along with local defibrillators are always accessible. The “code team” can be called from the UW Hospital as the WIMR building is continuously attached to the Hospital. For Waisman, 911 will be called in an emergency.
PI Lab: Screening and maximal fitness testing occurs in PI lab or Human Exercise Research Core (HERC) at School of Nursing. A defibrillator is available in the hallway near our laboratory. 911 will be called in an emergency.

7.8.1. Initial Screening

Phone screen or internet screen: All subjects will be asked to complete a screening (via Redcap survey) documenting physical activity, medications, and personal and family history of cardiovascular disease and risks. The questions are designed to immediately eliminate those subjects meeting exclusion criteria, and most importantly identify and exclude subjects who are at increased risk for IV catheter problems, adverse drug reactions, or MRI risk.

If the individual is deemed ineligible, then their screen information will be destroyed. If an interested individual is deemed initially eligible based off of this pre-screen, they will be invited to come in for a more thorough in-person screen visit. To maximize the likelihood of studying only appropriate subjects for this study (e.g. no body metal implanted for MRI scanning), and minimize the risk of bleeding or bruising in at risk subjects, a detailed relevant cardiovascular medical history will be performed prior to the catheterization procedure.

7.8.2. Subject Monitoring

Subject Monitoring: All testing will include subject monitoring of heart rate (HR) and blood pressure, to address subject stopping guidelines/safety and ensure data integrity. HR will be measured by Polar Heart Rate Monitor. Blood pressure will be monitored by a sphygmomanometer on the brachial artery. Oxygenation will be monitored on finger or ear SaO_2 monitor, and end tidal CO_2 by sampling of expired gases from mask/mouthpiece. With continuous monitoring, we can readily observe any adverse cardiovascular events and move to intervene immediately.

The frequency of monitoring is as follows: during baseline and each major MRI sequence (ASL or VIPR). Visually, HR, O_2 Sats and ETCO2 are continuous readouts. Practically speaking, all are recorded every 5-10 minutes on CRFs. These are monitored by trained laboratory staff who are physiologists-typically graduate students, postdoctoral fellows, or PI.

7.8.3. Blood Sampling

Blood sampling (not venipuncture or IV placement) will be performed only by research personnel that have had phlebotomy training. This training has encompassed the insertion, maintenance, and use of intravenous catheters. These personnel all have at least a bachelor’s degree or higher health and/or physiology-related education in terms of academic preparation. We feel education and training of personnel, and our positive track record over 15 years at UW performing similar invasive procedures, provide high confidence IV blood draws present a very low risk to subjects.

7.8.4. MRI

MRI risks will be minimized by thorough MRI screening at screening visit, and by MRI technicians immediately prior to MRI visit. Second, all MRI sequences fall under FDA approved guidelines and carry very small risks like muscle tingling described under MRI risks. Subjects have verbal contact with MRI

technicians and study team in control room, as well as a “panic button” they can press in case they feel any pain or discomfort.

7.8.5. Protecting the Confidentiality of Participant Data

This study is funded by the National Institutes of Health and is covered by a Certificate of Confidentiality.

Risks to confidentiality will be minimized by keeping copies of the documents linking study assignment number and the participant’s unique identifiers with the participant’s informed consent in locked cabinets of the offices of Dr. Bill Schrage. These linking documents will be destroyed, along with the data collection documents, seven years after the closure of the study. Only subject numbers will be used for group assignment, data processing, and analyses. Key linking identities (REDCap number) to Study ID (IRB#XXXX-M/F-XXX) will be stored separately from coded study data. All data will be stored in locked cabinets in a secure lab, and electronic files are all stored on password-protected databases and computers.

Personal information such as name, gender, date of birth, and medication history will be stored in a locked file cabinet in a locked office in the PI’s laboratory. Subject information will be coded to remove any personal identifiers during data analysis or research publications. Research oversight and regulatory groups may review study records.

Data will be stored as both hard copies and electronic files. Hard copy data collection forms will be locked in a file cabinet in the restricted-access laboratory area. Electronic files that contain potential Protected Health Information (PHI) will be stored in REDCap, secure web-based data management systems, and/or a Secure Box folder configured with the involvement of the UW-Madison HIPAA security officer. Electronic files will be HIPAA compliant and will use a subject ID instead of the subject’s name. The raw MRI data will be stored on servers assigned to Dr. Wieben and managed by the Departments of Medical Physics and Radiology and/or on encrypted external hard drives.

REDCap is Part 11 compatible but has not been validated for compliance. That may be completed as early as 2024. However, data can still be entered and stored in REDCap as long as source materials are available in hard copy format.

Blood samples may be analyzed for various hormones and inflammatory markers externally to the study team (e.g. UW Primate Research Center). Samples analyzed at research labs (e.g. UW Primate Research Center) will remain coded such that the outside blood analysis team will not be provided any identifiers or means by which they could connect the blood samples to any individuals that have participated in the study.

7.9. Stopping Guidelines

7.9.1. Study Stopping Guidelines

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to investigator, funding agency, and regulatory authorities. If the study is prematurely terminated or suspended, the PI will promptly inform the IRB and will provide the reason(s) for the termination or suspension. Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to subjects

- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable

The study may resume once concerns about safety, protocol compliance, and data quality are addressed and satisfy the applicable regulatory authorities.

7.9.2. Subject Stopping Guidelines

Participation in the study will be terminated based on the following:

1. If the subject wishes to stop

Each intervention (i.e. study visit) will be terminated based on the following specific guidelines:

1. If the subject wishes to stop
2. If the subject complains of serious pain or discomfort during study procedures
3. If the subject complains of severe anxiety or claustrophobia related to MRI
4. Any SAE occurring during study procedures

8. STATISTICAL CONSIDERATIONS

8.1. MRI Data Processing

Standard and validated tools will be used for image processing. The research team is expert in analyzing brain imaging data and have published several studies focused on MRI-derived blood flow-based measures.

Maps representing gray matter probability and cerebral blood flow will be generated using SPM, FSL, or similar software. Scans will be processed using the most up to date and recommended processing scheme, including methods previously used by our research group and collaborators.

Blood-oxygen-level-dependent (BOLD) fMRI data will be acquired during the two in-scanner tasks to localize the associated brain regions.

8.2. Statistical Summary

Statistical power calculations were performed in consultation with Biostatistician Dr. Ronald Serlin (long-time collaborator) Power for our primary outcomes in each Aim is based on n=15/group. Power to detect sex differences in CBF or changes with AOC the first of the sequential tests (power>0.8) using Shafer's sequential method. Shafer's sequential method provides greater power to detect sex differences in ΔCBF for any of five cerebral lobes/regions for the first of the sequential tests (alpha = .01) and to detect an effect of AOC is 0.75.

9. DATA AND RECORD KEEPING

9.1. Data Management

Delegated and appropriately trained study team members will be involved in data collection. All personnel who will be involved in data collection have completed appropriate Human Subjects Protection training or will before they engage in contact with subjects and subject data.

The Research Electronic Data Capture (REDCap) system is used to manage the data for this study. REDCap is a largely self-service, secure, web-based application for building and managing data collection forms. REDCap provides data management functionality by allowing the development of instruments and surveys to support data capture for research studies.

The REDCAP system used for this study is managed by ICTR. The ICTR REDCap support team will work with the investigator, statistician, and study team to ensure the relevant, applicable study data are collected and managed with restricted access using the software electronic data collection form instruments.

9.2. Assured Confidentiality

The raw MRI data will be stored on servers assigned to Dr. Wieben and managed by the Departments of Medical Physics and Radiology. The UW-Madison School of Education houses the Schrage Lab server and all electronic files will be coded to maintain HIPAA compliance and stored on servers/folders that are HIPAA compliant. Subject information and study tracking information will also be stored in REDCap, which is a secure site used for data management.

Any forms with PHI will be stored in locked cabinets inside the PI's secure laboratory. All data collected electronically will be password protected on a secure database/drive configured with the involvement of the UW-Madison HIPAA security officer. These database/drives include Research Drive, REDCap, OnCore, PHI BOX, and Schrage Lab encrypted hard drives that are located in the secure lab space. Electronic files will be HIPAA compliant and will use a subject ID instead of the subject's name. Only coded data will be used in data analysis. Any publications arising from this protocol will not include any personal identifying information or study code, thereby making the reported data completely de-identified.

9.3. Data Collection Methods

Data will be collected using REDCap, MRI, offline data collection software, data analysis software, and data collection forms. These files will consist of electronic DICOM, .dat, and Excel files, as well as hard copies of data collection forms for each subject.

All other study data referenced above will be collected using study visit checklists and data collection forms, most of which constitute both the Case Report Form (CRF) and the original source (i.e., the first and only place the data is manually written/recorded). These study specific forms will be developed and maintained with the assistance of the ICTR DMC for recording all necessary data for each subject. It is the PI's responsibility to ensure that these are properly, legibly, and fully completed and signed where appropriate. The CRFs are used to record study data and are an integral part of the study and subsequent reports. Therefore, the CRFs must be completed for each subject screened or enrolled according to the subject's source data on a per-visit basis. All study visit checklist and data collection forms will be retained in the subject research chart/file. All applicable data collected using the study visit checklists and data collection forms will be entered into electronic Case Report Forms (eCRFs) within REDCap.

When the study is complete, the applicable CRFs must be signed by the investigator to attest that it is an accurate and complete record.

9.4. Retention of Study Records

In compliance with UW-Madison regulations, study records will be for a minimum retained for seven years following final project close-out.

10. DATA INTEGRITY

Justification for building in flexibility:

Increasing flexibility provides time for participants to get all MRI visits completed, and to overcome unexpected technical delays that briefly prolong the study visit (see below) without increasing risks.

Impact of building in flexibility on data integrity:
Flexibility will not impact data integrity. In fact, it should greatly reduce participants from dropping out of study, such that overall study risks will be reduced by reducing the total number of subjects enrolled to achieve primary outcomes.
Flexibility will enhance likelihood of collecting all primary data once a participant is enrolled.

Maximizing visit flexibility is important for meeting the competing influences of: female menstrual cycle timing, study team availability, and MRI scheduling availability.

No flexibility steps below significantly increase risk beyond those described in the main study design.

To build in flexibility while maintaining subject safety and data integrity. It will not be considered a deviation if:

1) Screening visit: it will not be considered a deviation if:
    a. Participants must return to lab to complete successful venipuncture
    b. Participants must return to complete successful gas challenge familiarization visit
    c. Participants must return to complete successful pregnancy test
    d. Visit Reminder is sent less than 12 hours prior to gas familiarization or exercise visit
2) MRI Study Visit: it will not be considered a deviation if:
    a. We need to conduct extra scans as needed in order to complete primary outcome data collection. This is unlikely, but technical issues may cause the restart of any given scan.
    b. Study visit not completed due to technical (e.g., error in gas challenge mixing, MRI failure, or practical reasons (scan time ended). Subject can be rescheduled for a complete study visit at on a future date.

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